Expression of Indoleamine 2,3-Dioxygenase in Dermal Fibroblasts Functions as a Local Immunosuppressive Factor

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As a possible way of making a non-rejectable skin substitute, here, we ask the question of whether the expression of indoleamine 2,3-dioxygenase (IDO) selectively suppresses immune, but skin, cell proliferation. To address this question, a series of experiments in which adenovirus (Ad-IDO) infected IDO expressing dermal fibroblasts were co-cultured with different types of immune cells were carried out. The immune cells were then harvested and evaluated for propidium iodide (PI) positive cells by FACS analysis. TUNEL assay was also carried out to determine the apoptotic status of these cells. The results showed that the expression of IDO in dermal fibroblasts significantly induces apoptotic death of PBMC, $CD4^+$-, $CD8^+$- and B cell-riched primary lymphocytes, Jurkat cells, and THP-1 cells. IDO-mediated damage of immune cells was restored by an addition of tryptophan and IDO inhibitor. Using the same approaches, we also demonstrated that skin cells and endothelial cells are remarkably resistant to tryptophan-deficient environment. Furthermore, no significant difference in cell proliferation between Ad-GFP (control)- and Ad-IDO-GFP-infected either keratinocytes or fibroblasts, was found. The results of this study, therefore, suggest that the expression of IDO by dermal fibroblasts mediates immune cell damage and this may shed a new light toward developing a non-rejectable skin substitute in the future.

Key words: apoptosis/bystander cells/fibroblasts/indoleamine 2,3-dioxygenase (IDO)/tryptophan
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Extensive skin loss from a variety of conditions such as severe thermal injury is associated with significant functional morbidity and mortality (Cairns *et al*, 1993). In recent years, however, the overall mortality rate has been improved for patients suffering from burns due in part to the significant biotechnological advancements in skin replacement for wound closure (Cairns *et al*, 1993). *In vitro*, cultivation of keratinocytes with the support of a feeder layer of lethally irradiated 3T3 cells was initially introduced by Rheinwald and Green (1975). These investigators were later able to grow keratinocytes to confluency, which was suitable for grafting (Gallico *et al*, 1984). Although sheets of autologous keratinocytes are currently used in some burn centers to treat patients with large thermal injury (Morhenn *et al*, 1982; Thivolet *et al*, 1986), this has not been a routine procedure due to: firstly sheets of keratinocytes prepared from layers of cultured keratinocytes without matrix are very fragile and difficult to cultivate, secondly the rate of graft-take is relatively low (50%–60%), thirdly patients with large injuries do not have enough of uninjured skin to be used for cell culture, and lastly generating another wound increases the risk of an infection and development of hypertrophic scarring. Considering the fact that all of these factors are pervasive medical problems with far-reaching clinical and economic implications, utilizing an allogeneic and readily available skin substitute seems logical to overcome these problems. Therefore, a series of experiments have been designed and conducted by our laboratory to examine the benefit of using allogeneic skin cells expressing indoleamine 2,3-dioxygenase (IDO) as a local immunosuppressive factor.

IDO is a monomeric heme-containing enzyme that catalyzes the opening of the pyrrole ring of L-tryptophan to yield *N*-formylkynurenine, which rapidly degrades to give kynurenine (Higuchi and Hayaishi, 1967). Interferon (IFN)-γ is a strong inducer of IDO expression in cultured fibroblasts (Dai and Gupta, 1990a), macrophages (Carlin *et al*, 1989), dendritic cells (Hwu *et al*, 2000), and many cancer cell lines (Taylor and Feng, 1991). IDO is also induced rather poorly by LPS (Yoshida and Hayaishi, 1978), IFN-α, and IFN-β (Bianchi *et al*, 1988). It is demonstrated that IDO activity significantly increases in certain pathophysiologic conditions such as transplanted tumor cells (Takikawa *et al*, 1990), viral transfected lung (Yoshida *et al*, 1979), and viral transfected epithelial cells (Jacoby and Choi, 1994). The role of IDO in the survival of fetal allograft during pregnancy

Abbreviations: FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; GFP, green fluorescence protein; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; MOI, multiplicity of infection; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PI, propidium iodide; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin Nick end labelling

has also been explored. Munn *et al* (1998) reported that the expression of IDO in the placenta is crucial in the prevention of immunological rejection of the fetal allograft. These investigators suggested that proliferation of infiltrated T cells was inhibited by IDO, as it generates a tryptophan-deficient environment in the placenta. *In vitro* studies have also demonstrated that IDO expression by macrophages (Munn *et al*, 1999) and dendritic cells (Hwu *et al*, 2000) inhibits bystander lymphocyte proliferation.

Considering the protective role of IDO in maternal T cell-mediated rejection of allogenic fetuses (Munn *et al*, 1998; Mellor *et al*, 2001) and the success of IDO in prolonging the survival of pancreatic islet cells (Alexander *et al*, 2002), we hypothesize that IDO expression may function as a local immune suppressive factor to protect allogenic skin substitutes. In this study, we, therefore, constructed and used recombinant adenoviral vectors bearing either green fluorescent protein (GFP) as a marker or GFP-IDO genes to infect dermal fibroblasts. Co-culturing IDO genetically modified fibroblasts with different types of immune cells, we demonstrated a significant increase in damage of bystander human peripheral blood mononuclear cells (PBMC), $CD4^+$-, $CD8^+$-, and B cell-enriched lymphocytes, CD4-positive Jurkat cells, and THP-1 cells relative to those of controls. This bystander effect proved to be due to IDO inducing a tryptophan-deficient cell culture environment. In addition, considering the fact that IDO expression seems to affect only the infiltrated immune, but not the non-immune cells in these tissue environments, here, we also hypothesize that skin cells such as keratinocytes and fibroblasts would survive and proliferate in IDO generating tryptophan-deficient environment. Using the same approaches, in this study, we demonstrated that fibroblasts would survive even after 15 d of being cultured in a low tryptophan environment. Both keratinocytes and endothelial cells co-cultured with IDO-expressing cells were also viable up to 5 d examined. These results suggest that the expression of IDO by dermal fibroblasts could function as a local immunosuppressive factor for prevention of rejection of grafted skin substitutes.

Results

Adenoviral vector-infected cells expressed IDO and GFP proteins To distinguish the IDO-infected cells from uninfected cells and to evaluate the level of IDO expression in these cells, we employed an adenoviral vector bearing the GFP gene, as a reporter gene, to construct a human IDO plus GFP recombinant adenoviral vector. As shown in the schematic diagram Fig 1*A*, the CMV5 promoter drives the expression of either GFP in control or IDO plus the GFP in the recombinant IDO vector. Recombinant human IDO gene and its correct orientation has been confirmed by serial restriction enzyme digestions and mapping (data not shown). A PCR analysis of the viral DNA product confirmed the successful transfection of IDO recombinant gene in packaging 293 cells (Fig 1*B*). Microscopic evaluation of Ad-GFP- (Fig 1*Ca*) and Ad-IDO-GFP- (Fig 1*Cb*) infected 293 cells showed a bright green fluorescent stained, indicating GFP expression in these cells. To further validate the biological activity of IDO protein, the levels of tryptophan and its IDO-generated degraded product, kynurenine, in conditioned medium derived from Ad-IDO-infected and non-infected 293 cells, were evaluated by LC–MS. As shown in Fig 2, the level of tryptophan (panel *A*) was almost undetectable in conditioned medium obtained from Ad-IDO-infected cells relative to that of either non-infected or Ad-GFP-infected controls, whereas the level of kynurenine (panel *B*) was more than 20-fold higher in the same conditioned medium.

In a similar experimental setting, fibroblasts, keratinocytes, and endothelial cells were separately infected with mock or IDO recombinant adenovirus and evaluated for efficacy of transfection. As shown in Fig 3*A*, after 72 h post-infection, more than 90% keratinocytes and endothelial cells were successfully transfected and were positive for GFP protein. Fibroblasts, however, had lower efficacy of Ad-GFP-IDO transfection compared with the other two cell types used. The number of GFP-positive fibroblasts was about 30%–50% as evaluated by FACS analysis (data not shown). To confirm these findings, the expression of IDO protein in Ad-GFP-IDO-infected fibroblasts, keratinocytes, and endothelial cells harvested at 72 h post-infection was analyzed by western blot using rabbit polyclonal anti-human IDO. As shown in Fig 3*B*, an anti-IDO antibody immunorecognized a 42 kDa band in IDO recombinant adenovirus-infected cells. The size of protein was consistent with the expected molecular weight of human IDO (Dai and Gupta, 1990b). Consistent with IDO protein expression, a significant increase in the levels of kynurenine, used as an index for IDO activity, was found in only Ad-GFP-IDO-infected fibroblasts and keratinocytes (data not shown).

IDO expression mediates bystander immune cell apoptosis To examine the mediating role of IDO expression in bystander immune cell damage, total human PBMC, $CD4^+$-, $CD8^+$-, and B cell-enriched cells as well as $CD4^+$ Jurkat cells, THP-1 monocytes as non-IDO-expressing bystander cells, were employed. In these experiments, IDO-infected human dermal fibroblasts were used as IDO-expressing cells, and non-adenoviral-infected and Ad-GFP-infected fibroblasts were used as controls. After 30 h of fibroblast infection and following confirmation of IDO and GFP expression on day 4 post-infection, cells were co-cultured with immune cells. After 3–5 d, bystander co-cultured immune cells were then harvested and evaluated for PI staining by FACS analysis. As shown in Fig 4*A*, almost all immune cells used are sensitive to IDO-induced low tryptophan environment. The highest number of PI-positive cells was found in the $CD4^+$ lymphocyte population (30%) relative to either PBMC (18%), $CD4^+$- (16%), and B cell-enriched (16.4%) population. The number of PI-positive immune cells was the same when they were co-cultured with either non- or AD-GFP-infected cells. Similarly, the number of PI-positive Jurkat and THP-1 cells was more than 16-fold (82.7% *vs* 5.2%) and 8-fold (35.8% *vs* 3.9%), respectively, higher when cells co-cultured with Ad-IDO-infected fibroblasts compared with those co-cultured with mock-infected fibroblasts (Fig 4*B*).

To further demonstrate that IDO induction is the main cause of bystander immune cell damage, IFN-γ as a strong

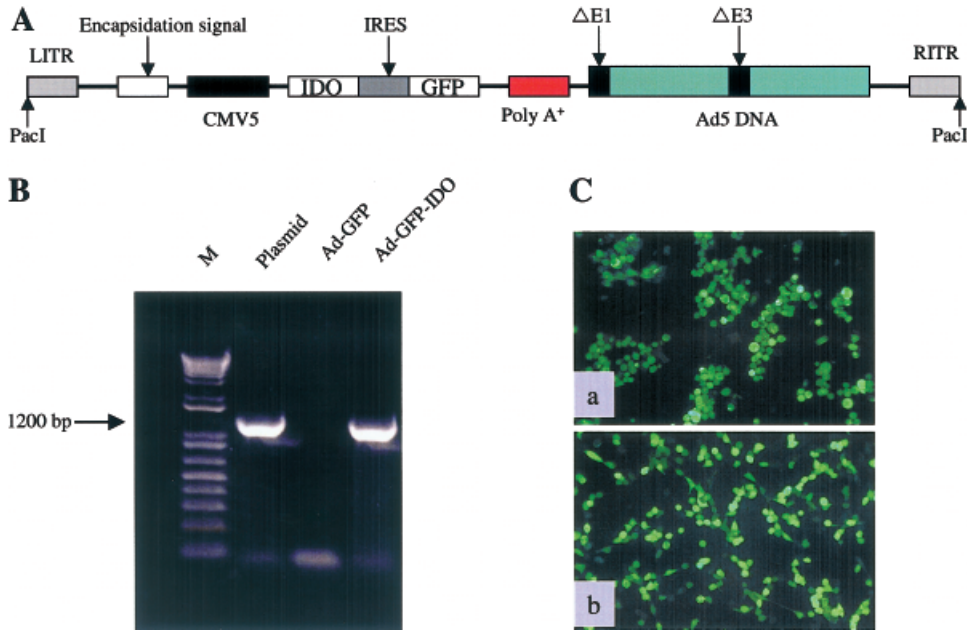


Figure 1
Schematic diagram of constructed human IDO, its PCR gene product and microscopic appearance of GFP expression. IDO was initially cloned into a shuttle vector, pAdenoVator-CMV5-IRES-GFP. Constructed plasmid was then linearized by *Pme*I restriction endonuclease and inserted into an adenoviral backbone plasmid, pAdenoVator ΔE1/ΔE3, and used to transform *Escherichia coli* BJ5183 cells (panel *A*). Finally, the linearized recombinant IDO plasmid was used to transfect adenoviral packaging 293 cells using Fugene-6 transfection kit. After 10 d, a high titer of IDO adenoviral particles was confirmed in 293 cells and the conditioned medium of transfected cells was used to infect the other cell strains. Panel *B* shows the PCR product detected in IDO-infected 293 cells using PCR procedure and specific IDO primers described in the Materials and Methods. Panel *C* shows microscopic appearance of GFP expression in 293 cells infected at a multiplicity of infection of 5 with either Ad-GFP (panel *Ca*) or Ad-GFP-IDO (panel *Cb*) for 3 d. IDO, indoleamine 2,3-dioxygenase; GFP, green fluorescent protein.

IDO inducer (Dai and Gupta, 1990a) was used to treat dermal fibroblasts as a source of IDO induction. The induction of IDO mRNA expression was confirmed by northern analysis; whereas the activity of IDO was monitored by measuring the level of kynurenine in condition medium by LC–MS (data not shown). After confirmation of IDO induction and activity, fibroblasts were co-cultured with $CD4^+$ Jurkat cells for another 4 d. Unattached Jurkat cells were then harvested and stained with PI. The number of PI-positive cells were evaluated by FACS analysis. As shown in Fig 4*C*, the number of PI-positive Jurkat cells co-cultured with IFN-γ pre-treated fibroblasts increased more than 25-fold (77% *vs* 3%) relative to those co-cultured with IFN-γ untreated fibroblasts. This result thus provides further evidence that IDO expression causes bystander immune cell damage.

To investigate whether the mediating role of IDO in bystander immune cell damage is due to apoptosis, PI staining, TUNEL assay and nuclear fragmentation were used. The results of the microscopic PI-stained cells revealed a typical nuclear fragmentation, a hallmark of apoptosis, in Jurkat cells co-cultured with IDO-expressing fibroblasts for 3 d (Fig 5*A*). Consistent with PI staining results, the finding of TUNEL assay demonstrated a large number of apoptotic Jurkat cells co-cultured with either IFN-γ pre-treated fibroblasts (Fig 5*Bb*) or Ad-IDO-infected fibroblasts (Fig 5*Cc*). In contrast, no sign of apoptotic cells was found in Jurkat cells co-cultured with either non-infected (Fig 5*Ba* and *Ca*) or Ad-GFP-infected (Fig 5*Cb*) cells. Furthermore, a typical DNA ladder, as another index for apoptosis, was also seen in Jurkat cells co-cultured with IDO-expressing fibroblast (data not shown). These results collectively suggest that IDO expression is responsible for the apoptosis of bystander immune cells in our co-cultured system.

Addition of tryptophan and IDO inhibitor restored immune cell damage induced by IDO To test the possible mechanism through which IDO mediates bystander cell damage, we examined the effect of tryptophan and its degraded product, kynurenine to see whether the expression of IDO-induced immune cell damage is due to kynurenine toxicity or tryptophan depletion. The results showed that an addition of kynurenine at various concentrations ranging from 40 to 400 μM to the conditioned medium of cultured THP-1 cells and Jurkat cells has no cytotoxic effect on these cells (data not shown). But an addition of L-tryptophan to conditioned medium of either of these cell types markedly decreased bystander cell damage. As shown in Fig 6, the number of PI-positive Jurkat cells and THP-1 co-cultured with IDO-expressing fibroblasts markedly reduced, from 83% to 13% and 33% to 3%, respectively. To confirm these results, a specific IDO inhibitor, L-methyl D-tryptophan, was added to conditioned medium at a concentration of 800 μM. As expected, the number of PI-positive Jurkat and THP-1 cells co-cultured with IDO-expressing fibroblasts markedly reduced from 82% to 25% and from 33% to 5.4%, respectively.

Skin cells and endothelial cells are resistant to IDO-induced low tryptophan culture environment To assess the mediating role of IDO expression in bystander primary

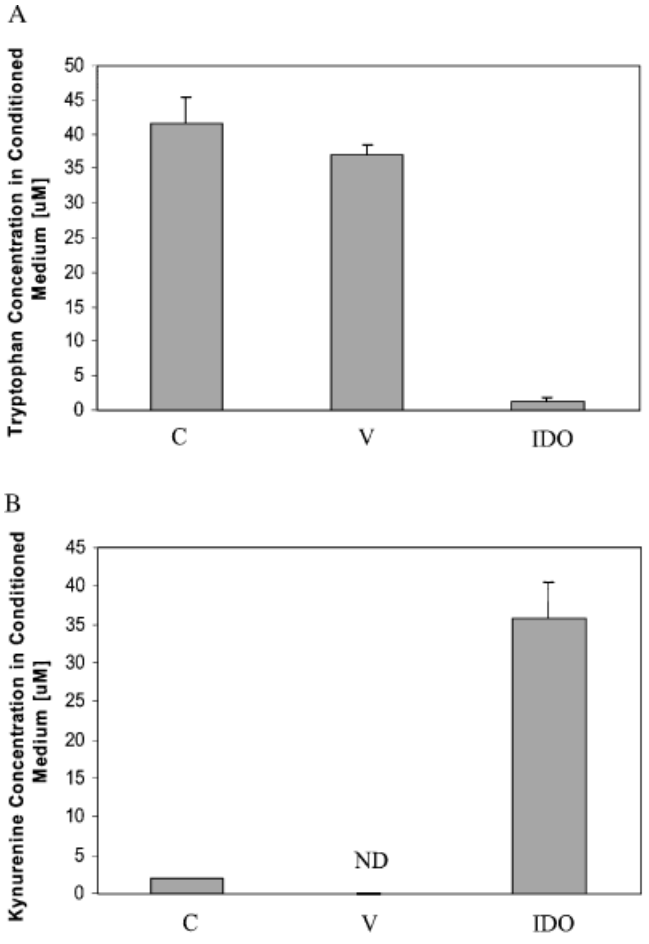


Figure 2
Tryptophan and kynurenine levels in conditioned medium of cultured 293 Cells with or without recombinant adenoviral infection. To examine the IDO enzyme activity, the conditioned medium derived from either non-infected, Ad-GFP-, or Ad-IDO-GFP-infected cells was collected and evaluated for the levels of either tryptophan (panel *A*) or kynurenine (panel *B*) using the liquid chromatography with electrospray mass spectrometry procedure described in the Materials and Methods. IDO, indoleamine 2,3-dioxygenase; GFP, green fluorescent protein.

skin cells and endothelial cells, either Ad-GFP- or Ad-GFP-IDO-infected keratinocytes, endothelial cells (Fig 7*A*), and fibroblasts (Fig 7*B*) were co-cultured with its corresponding non-infected cells, respectively. Cells from the bottom chambers (non-infected cells) were harvested and stained with PI. Positive cells were determined by FACS analysis. As shown in Fig 7*A*, only 2.6% of keratinocytes grown in IDO-induced tryptophan depleted medium were PI positive after 5 d of culture. Similarly the number of PI-positive endothelial cells (Fig 7*A*) and fibroblasts (Fig 7*B*) grown in the same environment for 5 d was less than 3.5%. When this duration was extended to 15 d for dermal fibroblasts, no significant difference in numbers of PI-positive fibroblasts between Ad-GFP- and Ad-GFP-IDO-infected cells was found (Fig 7*B*). These results reveal that primary skin cells such as keratinocytes and fibroblasts as well as endothelial cells are resistant to IDO-generated low tryptophan environment, at least, for the indicated durations examined.

IDO does not alter proliferative capacity of infected fibroblasts and keratinocytes To examine whether IDO expression influences the proliferative capacity of IDO-expressing cells, either fibroblasts (Fig 8*A*) or keratinocytes (Fig 8*B*) were infected with either nothing (column C), Ad-GFP (column V), or Ad-GFP-IDO (column IDO). Cell proliferation was then evaluated by [^{3}H]thymidine incorporation after 3 and 6 d post-infection. As shown in Fig 8*A*, infected fibroblasts showed less [^{3}H]thymidine incorporation at both time points examined relative to those of non-infected cells (column C, $p < 0.05$). Similarly, infected keratinocytes (Fig 8*B*) also showed less [^{3}H]thymidine incorporation at day 3 ($p < 0.05$). The rate of cell proliferation between infected and non-infected keratinocytes, however, did not show a significant change at day 6 ($p > 0.05$). Compared with Ad-GFP-infected fibroblasts or keratinocytes, proliferation of Ad-GFP-IDO-infected fibroblasts was higher at both time points ($p < 0.05$) and higher for IDO-expressing keratinocytes at day 3 ($p < 0.05$). These results may imply that adenovirus, but not IDO expression, is responsible for the suppression of IDO-expressing fibroblast and keratinocyte proliferation.

Discussion

Considering the fact that an ultimate goal of any tissue engineering related study is to explore the possible approaches through which the clinical complications of both non-healing and over-healing wounds are improved, there is a need to develop a non-rejectable and readily available skin substitute. This skin substitute would function as a wound coverage to prevent or reduce heat and fluid loss, and to prevent wound infection. The skin substitute should also function as a wound coverage through which dermal–epidermal wound-healing modulating factors are released locally to facilitate granulation tissue formation, re-epithelialization, and improve closure of non-healing wounds, such as those seen in the diabetic patients. Different approaches have been attempted for temporary or permanent coverage of wounds. Synthetic dressings (e.g. Integra and Dermagraft artificial skin) and biological dressings (e.g. Alloderm and Apligraf artificial skin) have been developed for the last 30 y (Philips 1998). Two products with allogeneic components are commercially available. Dermagraft consists of living allogeneic dermal fibroblasts grown on degradable scaffold. Apligraf is a living allogeneic bilayered construct containing keratinocytes, fibroblasts, and bovine collagen. Although, these skin substitutes have not been shown to be immunogeneic by limited clinical data, the potential risks of acute and chronic rejection of allograft need to be further evaluated (Briscoe *et al*, 1999; Badiavas *et al*, 2002). Previous studies have shown that even in the absence of passenger leukocytes and langerhans cells, cultured allogeneic skin cells used as a wound coverage were rejected (Aubock *et al*, 1988; Phillips, 1991; Rouabhia *et al*, 1993). Furthermore, a recent study by Lamme *et al* (2002) demonstrated that allogeneic fibroblasts in a dermal skin substitute induce inflammation and scar formation. Engineered skin substitutes using the patient's skin cells are far more desirable. But it is difficult to perform an autologous engraftment for patients who suffer from extensive skin loss from a variety of conditions such as

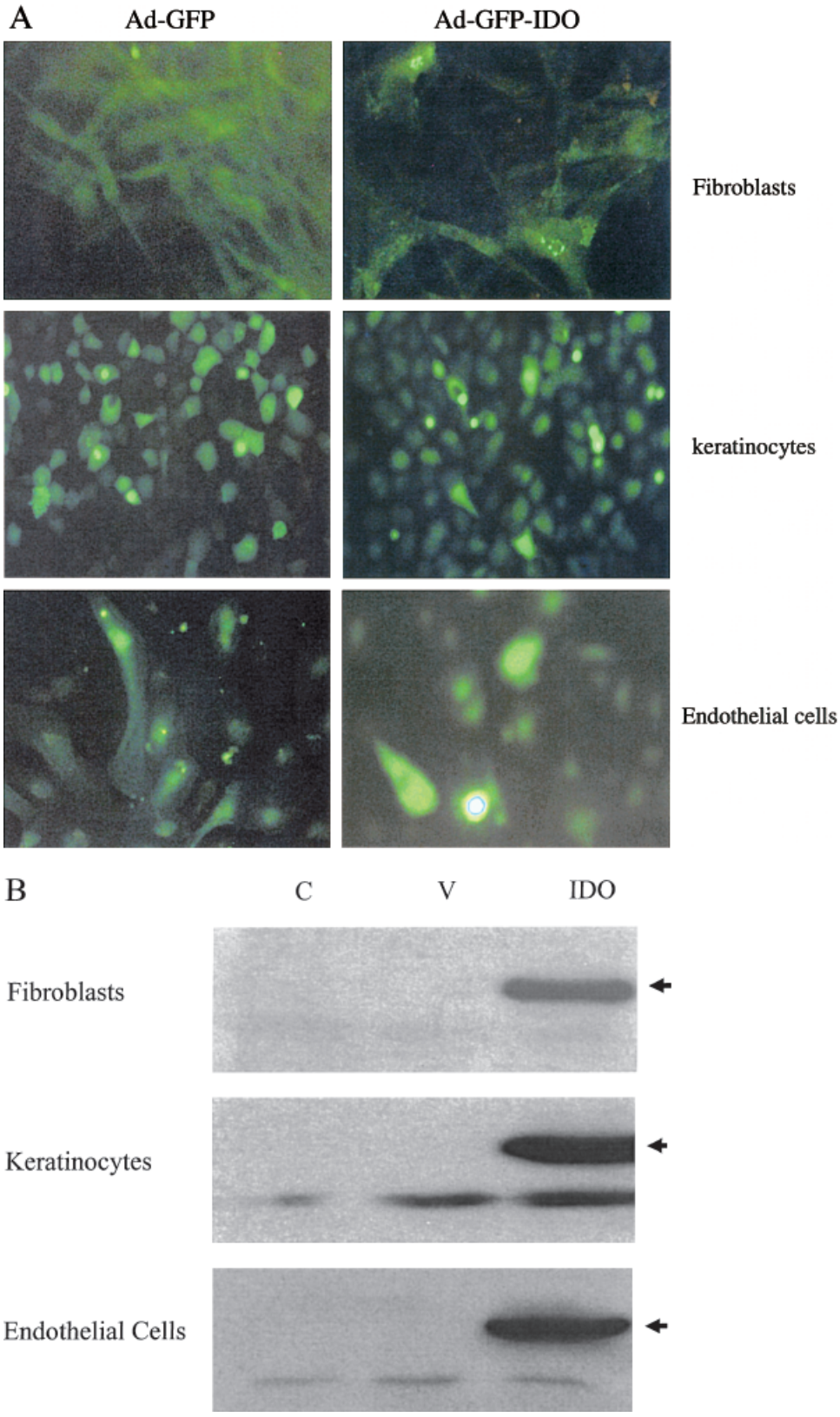
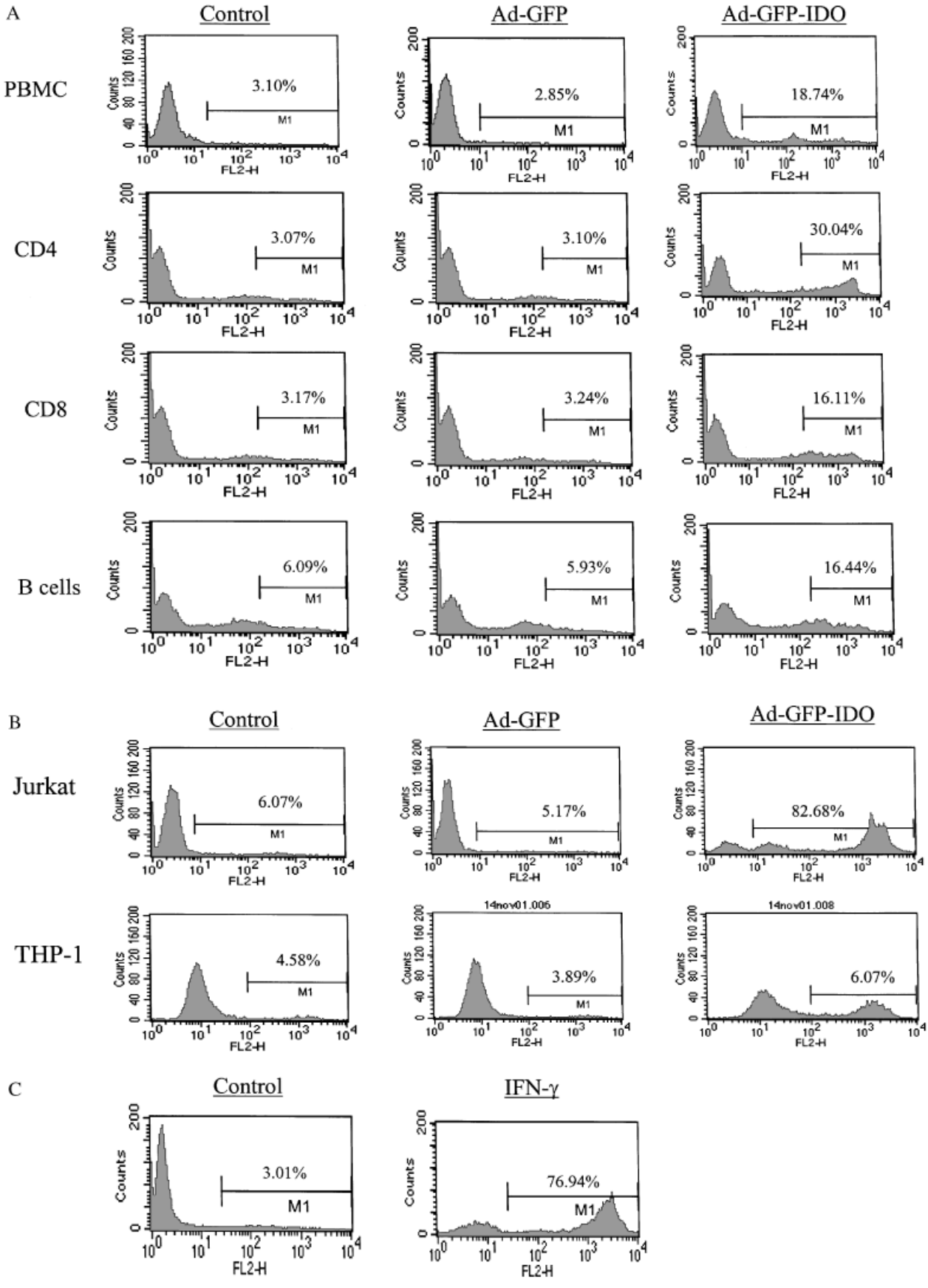


Figure 3
Efficiency of IDO transfection in fibroblasts, keratinocytes and endothelial cells using an adenovirus recombinant system (*A*) GFP expression in fibroblasts (upper panel), keratinocytes (medium panel), and endothelial cells (lower panel) after 72 h infection with either Ad-GFP (left panel) or Ad-IDO-GFP (right panel) . Fibroblasts, keratinocytes, or endothelial cells were infected with either Ad-GFP or Ad-IDO-GFP at indicated multiplicity of infection of 2000, 100, 100, respectively. The extent of infection was monitored by GFP expression under fluorescence microscopy. Magnification $\times$ 100. (*B*) Western blot analysis of IDO expression. Fibroblasts, keratinocytes, or endothelial cells were infected with either Ad-GFP or Ad-IDO-GFP for 72 h. Non-infected and infected cells were harvested, and cell lysates were fractionated by SDS-PAGE. IDO protein was detected using a polyclonal antibody from rabbit immunized with a purified recombinant IDO protein. Arrow shows a 42 kDa band corresponding to the IDO protein. GFP, green fluorescent protein; IDO, indoleamine 2,3-dioxygenase.

large and severe thermal injury due to limited amount of uninjured tissue. This is also true for diabetic, elderly, and immune-compromised patients who suffer from non-healing complications. Therefore, exploring an allogeneic, non-rejectable, and readily available skin substitute may provide a better means of improving wound healing. As rejection is a major obstacle in any type of grafting, this study seeks a novel approach through which local induction of immunosuppressive factors, such as IDO, a tryptophan catabolizing enzyme, generates a tryptophan-deficient microenvironment in which immune cells are unable to survive.


A
Control
Ad-GFP
Ad-GFP-IDO
PBMC
3.10%
2.85%
18.74%
CD4
3.07%
3.10%
30.04%
CD8
3.17%
3.24%
16.11%
B cells
6.09%
5.93%
16.44%
B
Control
Ad-GFP
Ad-GFP-IDO
Jurkat
6.07%
5.17%
82.68%
THP-1
4.58%
14nov01.006
3.89%
14nov01.008
6.07%
C
Control
IFN-γ
3.01%
76.94%
M1
Counts
FL2-H

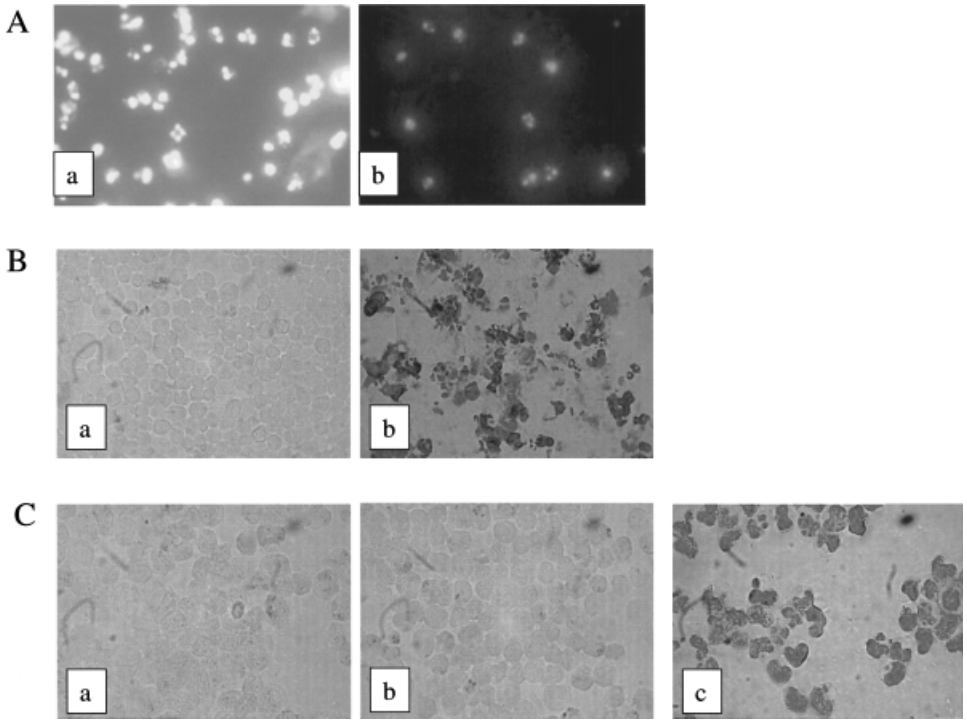


Figure 5
PI-positive cell microscopic appearance and apoptosis of bystander immune cells. To evaluate nuclear fragmentation of apoptotic bystander immune cells (panel *A*), IDO-expressing cells were co-cultured with Jurkat cells for 3–4 d. Jurkat cells were then harvested and stained with 10 μg per mL of PI. The PI-positive cells were visualized under fluorescent microscope (original magnification × 400). Panels *Aa* and *Ab* are Jurkat cells co-cultured with either IFN-γ-treated fibroblasts or Ad-GFP-IDO transfected fibroblasts, respectively. For TUNEL assay, Jurkat cells were co-cultured with either non-treated fibroblasts (panel *Ba*) or IFN-γ pre-treated fibroblasts (panel *Bb*) for 4 d. Junkat cells were harvested and then subjected to TUNEL assay to determine the apoptotic cells. In a similar experiment (panel *C*), Jurkat cells were co-cultured with either non-infected (*a*), Ad-GFP-infected (*b*), or Ad-GFP-IDO-infected (*c*) fibroblasts for 3 d and then subjected to TUNEL assay. PI, propidium iodide; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; GFP, green fluorescent protein; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin Nick end labelling.

As a part of a series of experiments, here, we constructed two human adenoviral vectors bearing either GFP as a reporter gene or IDO plus GFP genes and demonstrated that dermal fibroblasts can successfully be infected and the resultant IDO is catalytically active in degradation of tryptophan, an essential amino acid required for protein synthesis. In a co-culture system, the IDO expression in dermal fibroblasts generated a tryptophan-deficient environment in which the majority of immune cells such as PBMC, $CD4^+$, $CD8^+$, B cells, Jurkat cells, and THP-1 monocytes were unable to survive. As the same adenoviral vector (Ad-GFP vector) was used as a negative control, any alteration in immune cell damage should be due to the expression of IDO itself. The number of PI-positive Jurkat cells co-cultured with IFN-γ pre-treated fibroblasts increased more than 25-fold relative to those co-cultured with IFN-γ non-treated fibroblasts. This result provides further evidence that it is IDO expression and not adenoviral components that causes bystander immune cell damage.

A role for cell expressing IDO in inhibiting immune responses *in vivo* is consistent with that previously reported in murine pregnancy, grafted IDO-expressing islets, and IDO-expressing tumor cells injected into mice (Munn *et al*, 1998; Alexander *et al*, 2002; Mellor *et al*, 2002). In this regard, it has been hypothesized that IDO expression by either trophoblasts and macrophages in placenta, or IDO-infected islets and tumor cells, helps to maintain peripheral tolerance through regulating allogeneic T cell response. Indeed, *in vitro* studies showed that monocyte-derived macrophages exposed to macrophage colony-stimulating factor (MCSF) acquire the capacity to suppress T cell proliferation by inducing IDO expression (Munn *et al*, 1999). Similarly, Hwu *et al* (2000) reported that the proliferation of OKT3-stimulated autologous T cells is also inhibited by IDO producing dendritic cells. These, as well as similar findings obtained with IDO recombinant adenovirus transduced dendritic cells (Terness *et al*, 2002) support the hypothesis that IDO expression by antigen-presenting cells is responsible for suppression of "unwanted" T cells. During pregnancy, however, IDO expression is restricted to the maternal–fetal interface (Sedlmayr *et al*, 2002). Similarly, the expression of IDO in genetically modified islets and tumor cells is also found locally. These findings suggest that local expression of IDO by skin cells is likely to function as a local immunosuppressive factor for infiltrated immune cells at the wound site. Others have suggested that tryptophan deprivation causes activated T cell apoptosis induced by expression of FasL (Lee *et al*, 2002). It is also reported that a tryptophan metabolite, 3-hydroxyanthranilic acid induces THP-1 and U937 cell apoptosis (Morita *et al*, 2001). Collectively, these results indicated that bystander immune

Figure 4
FACS analysis of PI positive bystander immune cells. (*A*) Non-viral-infected (control) and pre-infected fibroblasts with either an empty vector (Ad-GFP) or IDO adenoviral vector (Ad-GFP-IDO) were co-cultured with either human PBMC, $CD4^+$-, $CD8^+$-, or B cell-enriched immune cells for 5 d, respectively. The immune cells were then harvested and stained with 10 μg per mL of PI for 10 min and analyzed by FACS. (*B*) Non-viral-infected (control) and pre-infected fibroblasts with either an empty vector (Ad-GFP) or IDO adenoviral vector (Ad-GFP-IDO) were co-cultured with either $CD4^+$ Jurkat cells or THP-1 monocytes for 3 d, respectively. The bystander immune cells were then harvested and stained with 10 μg per mL of PI for 10 min and analyzed by FACS. (*C*) $CD4^+$ Jurkat cells were co-cultured with either non-treated fibroblasts or IFN-γ pre-treated fibroblasts for 4 d. Jurkat cells were then harvested and stained with 10 μg per mL of PI for 10 min and analyzed by FACS. FACS, fluorescence-activated cell sorting; PI, propidium iodide; GFP, green fluorescent protein; IDO, indoleamine 2,3-dioxygenase; PBMC, peripheral blood mononuclear cells; IFN, interferon.

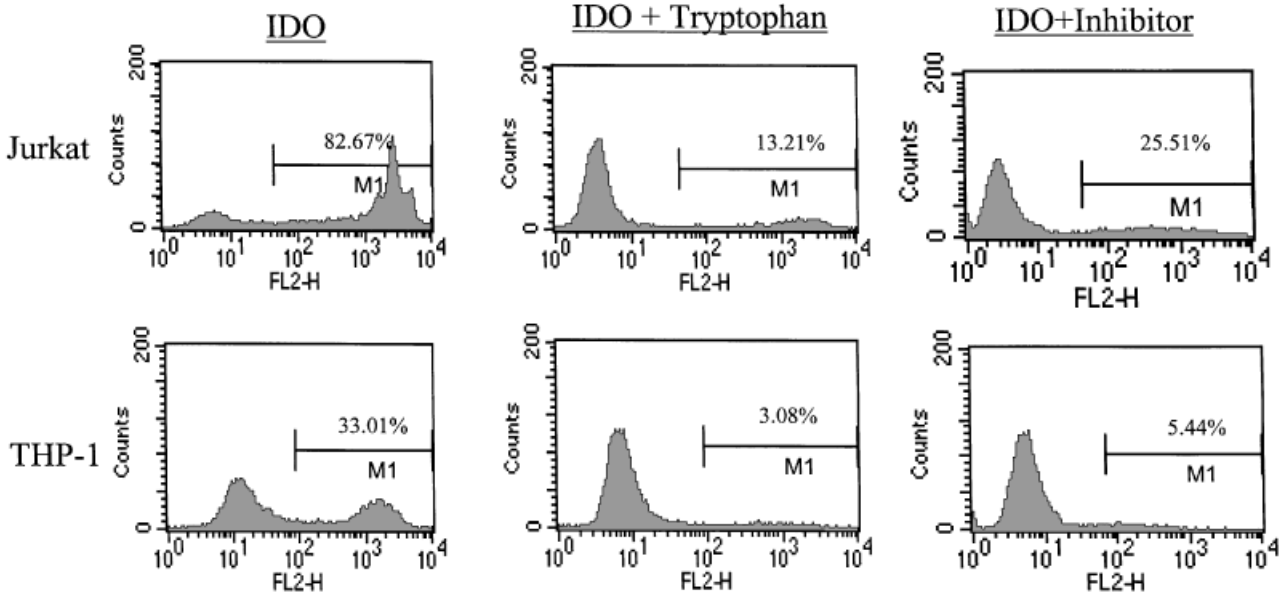


Figure 6
Addition of tryptophan prevents the IDO-induced apoptosis of bystander cells. Jurkat cells (panel *A*) or THP-1 monocytes (panel *B*) were co-cultured with Ad-IDO-infected fibroblasts in the presence (right panel) and absence (left panel) of 250 μM of L-tryptophan for 3 d. The immune cells were then harvested, stained with 10 μg per mL of PI, and the number of PI-positive cells was determined by fluorescence-activated cell sorting analysis. IDO, indoleamine 2,3-dioxygenase; PI, propidium iodide.

cell damage induced by IDO maybe a key contributor for IDO mediating immune tolerance.

To gain insights into the mechanisms of IDO-induced apoptosis of immune cells, we evaluated the effects of an addition of tryptophan and its catabolite, kynurenine, in conditioned medium and demonstrated that an addition of tryptophan restored the bystander effects of IDO expression for co-cultured immune cells. In contrast, an addition of kynurenine to conditioned medium failed to induce immune cell damage. This is consistent with previous studies indicating that depletion of tryptophan is involved in human IFN-γ-mediated apoptosis (Konan and Taylor, 1996). Furthermore, we have demonstrated that IDO inhibitor at a concentration previously reported (Sarkhosh *et al*, 2003) also prevents the mediating effect of IDO in bystander cell damage. Collectively, these results suggest that depletion of tryptophan, but not kynurenine toxicity, is the main mechanism by which IDO expression induces bystander immune cell damage.

Although, we have demonstrated that IDO-mediated activated immune cell damage via tryptophan depletion of culture environment, the degree of cell sensitivity to low tryptophan environment seems to vary from one cell strain to another. As shown in Fig 4, the number of PI-stained $CD4^+$ Jurkat cells co-cultured with IDO-expressing fibroblasts was markedly higher than those of THP-1 monocyte cells. Similarly, $CD4^+$-enriched lymphocytes were more sensitive to a tryptophan-deficient environment compared with $CD8^+$- and B cell-enriched lymphocyte population. It is not clear why there is a great variation in sensitivity of these immune cells to low tryptophan environment. It seems that high proliferating immune cells such as Jurkat cells are more sensitive to tryptophan-deficient environment than other cells. It should be emphasized that primary skin cells such as dermal fibroblasts, keratinocytes, and endothelial cells seem to be more resistant to low tryptophan than immune cells (Fig 7). As the success of making a non-rejectable skin substitute depends on skin cell survival in IDO-generated low tryptophan environment, it would be encouraging to see that primary skin cells, but not immune cells, are resistant to an IDO-induced tryptophan-deficient environment. Consistent with this data, our preliminary *in vivo* experimental data using a fibroblast embedded collagen as wound coverage clearly showed that the tryptophan-deficient environment generated by IDO is not toxic to the normal wound-healing processes. Surprisingly, grafting IDO-expressing fibroblasts embedded collagen gel would accelerate wound healing and closure compared with untreated wounds, untreated fibroblasts and adenoviral vector-infected fibroblasts embedded collagen gel (data not shown). The feasibility of usage of IDO-infected skin cells embedded collagen gel as wound coverage is under our current investigation.

In conclusion, we constructed an adenoviral vector bearing IDO gene and for the first time demonstrated that dermal fibroblasts expressing IDO generated a tryptophan-deficient microenvironment in which majority of immune cells were unable to survive. Further we also showed that primary skin cells are less sensitive to IDO-generated tryptophan-deficient environment. This differential sensitivity seen between immune and primary cells would make our attempt easier in preparing a non-rejectable allogeneic skin substitute to be used not only as wound coverage but also as a rich source of wound-healing promoting factors. Thus, the finding of this study just initiates a new approach through which the feasibility and benefit of a local immunosuppressive factor such as IDO in development of a non-rejectable skin substitutes is evaluated.

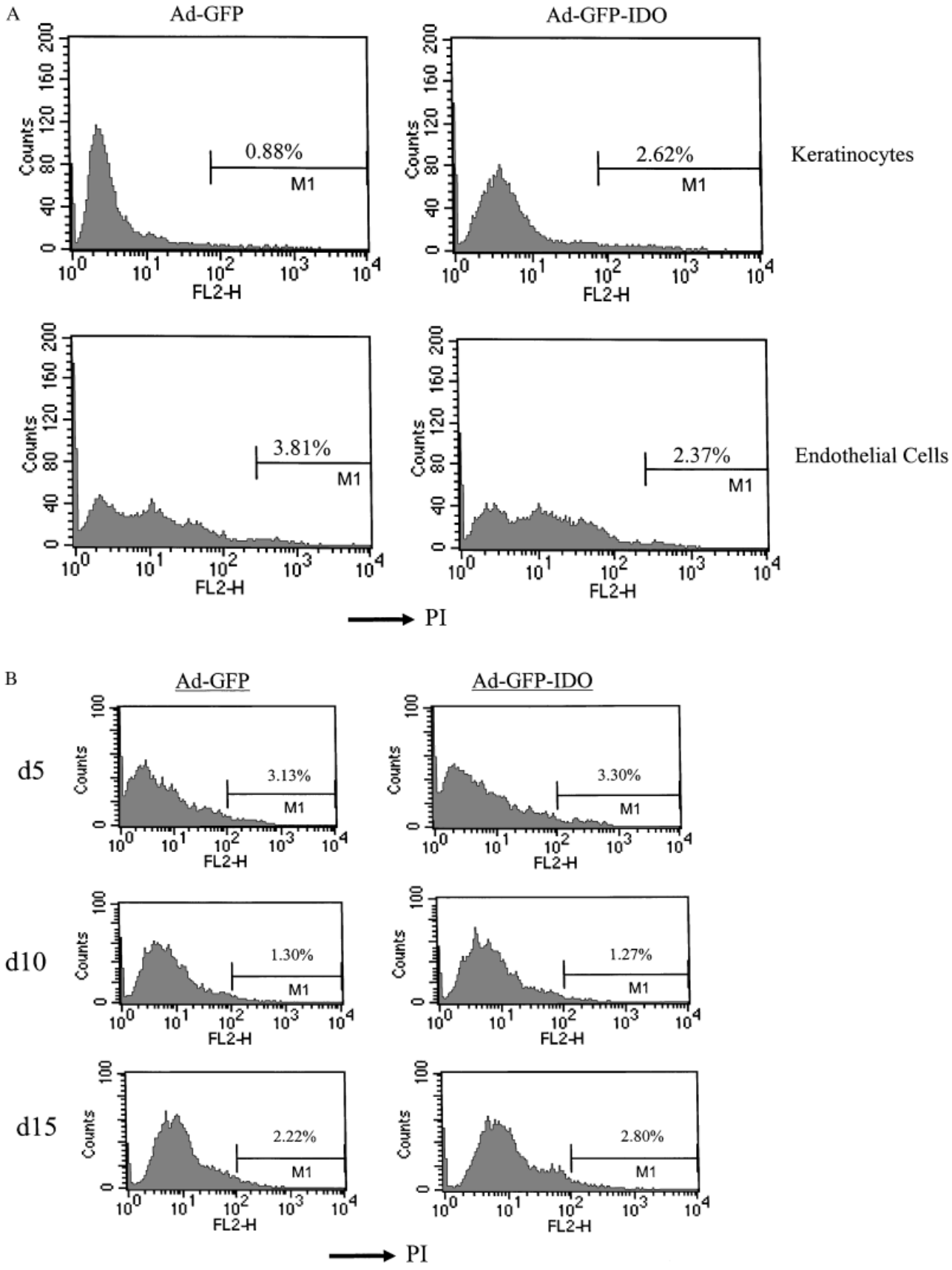


Figure 7

FACS analyses of PI-positive bystander skin cells and endothelial cells. Either keratinocytes (panel *A*, upper panel) or endothelial cells (panel *A*, lower panel) were co-cultured with either Ad-GFP- (left) or Ad-IDO-GFP- (right) infected cells for 5 d in a two-chamber co-culture system. The non-infected cells from bottom chambers were harvested and stained with PI. FACS analysis was used to determine the number of PI-positive cells. In the similar experimental conditions, Foreskin human fibroblasts (panel *B*) were co-cultured with pre-infected fibroblasts by either Ad-GFP control (left) or Ad-IDO-GFP (right) for 5, 10, and15 d. The non-infected fibroblasts were harvested and stained with PI for FACS analysis. FACS, fluorescence-activated cell sorting; PI, propidium iodide; GFP, green fluorescent protein; IDO, indoleamine 2,3-dioxygenase.

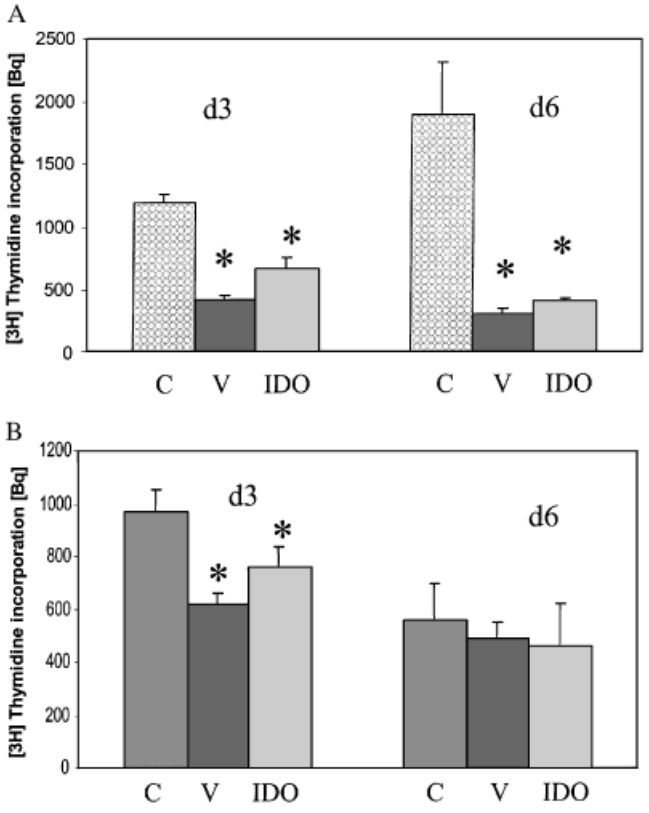


Figure 8
Effect of IDO on proliferative capacity of fibroblasts and keratinocytes. Fibroblasts (panel *A*) were infected with either Ad-GFP (V) or Ad-IDO-GFP (IDO) at multiplicity of infection (MOI) of 2000. After 30 h, free viral particles were removed by washing with phosphate-buffered saline and incubated up to day 3 or 6 post-infection. After addition of 1 μCi per mL of [^{3}H]thymidine, cell proliferation was determined and compared with the proliferation of non-infected control cells (C). Similarly, keratinocytes (panel *B*) were infected with either Ad-GFP or Ad-IDO-GFP at MOI of 100 for 30 h. Cell proliferation was determined at days 3 and 6. Each graph is a representative experiment of four repeats. Values presented are means ± SD for four samples. $^*p < 0.05$, control (C) *versus* either vector alone (V) or Ad-GFP-IDO (IDO). IDO, indoleamine 2,3-dioxygenase; GFP, green fluorescent protein.

Materials and Methods

Immune and non-immune cell cultures Following informed consent, skin punch biopsies were obtained from patients undergoing elective reconstructive surgery, under local anesthesia, according to a protocol approved by the University of Alberta Hospitals Human Ethics Committee. Biopsies were collected individually and washed three times in sterile Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Technologies, Carlsbad, CA) supplemented with antibiotic–antimycotic preparation (100 U per mL penicillin, 100 μg per mL streptomycin, 0.25 μg per mL amphotericin B) (Invitrogen). Cultures of fibroblasts were established as previously described (Karimi-Busheri *et al*, 2002). Upon reaching confluence, the cells were released by trypsinization, split for subculture at a ratio of 1:6, and reseeded onto 75 cm^2 flasks. Fibroblasts at passages three to seven were used in all experiments conduced in this study.

To establish cultured keratinocytes, the procedure of Rheinwald and Green (1975) was used. Human foreskin keratinocytes were grown using serum-free keratinocyte medium (KSFM, Invitrogen) supplemented with bovine pituitary extract (50 μg per mL) and EGF (5 μg per mL).

Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase digestion (Narumiya *et al*, 2001) and grown on gelatin-coated flask in M199 medium (Invitrogen) supplemented with 20% fetal bovine serum (FBS) and endothelial cell growth supplement (ECGS) (VWRCanlab, Mississauga, ON, Canada) at a final concentration of 10 μg per mL.

The Jurkat cells (ATCC) and THP-1 (ATCC) were maintained in RPMI 1640 containing 10% FBS and 2 mM glutamine. Human PBMC were isolated from donor blood by Ficoll-paque Plus (Amersham/Pharmacia Biotech, QC, Canada) and cultured in RPMI 1640 containing 10% FBS and 2 mM glutamine. Either CD4$^+$-, CD8$^+$-, or B cell-enriched lymphocyte population was purified by RosetteSep Cell Enrichment Kit (Stem Cell Technologies, Vancouver, BC, Canada). In brief, 4 mL of whole blood was incubated with the antibody-based enrichment cocktail at room temperature for 20 min. The RosetteSep antibody cocktail cross-linked unwanted cells in human whole blood to multiple red blood cells, forming immunorosettes. Following a standard buoyand density separation, either CD4$^+$-, CD8$^+$-, or B cell-enriched lymphocytes were harvested, washed by phosphate-buffered saline (PBS), and cultured in RPMI 1640 containing 10% FBS and 2 mM glutamine. All these cells were cultured at 37°C in an atmosphere of 5% CO_2 and 95% air. All media contained 100 U per mL penicillin and 100 U per mL streptomycin.

Construction of IDO adenoviral vectors and measurement of IDO enzyme activity Full-length human IDO gene was kindly provided by Dr J. M. Carlin (Department of Microbiology, Miami University, Oxford, Ohio). Following amplification by PCR, the IDO gene was subcloned into a shuttle vector containing a GFP gene according to manufacturer's instructions (Q-Biogene, Carlsbad, CA). The cloned plasmid was then homologously recombined with adenoviral plasmid in *Escherichia coli*, BJ5183, by electroporation. The success of IDO insertion into adenoviral plasmid was confirmed by restriction endonuclease mapping. Plasmid DNA was amplified in competent DH5α bacteria and purified by CsCl gradient in an ultracentrifuge. Adenoviral vectors carrying either GFP alone or GFP plus IDO gene were linearized by *Pac*I digestion and used to transfect 293A package cells using Fugene-6 transfection reagent (Roche, Laval, OC, Canada). Infected cells were monitored for GFP expression and after three cycles of freezing in an ethanol/dry ice bath and rapid thawing at 37°C, the cell lysates were used to amplify viral particles in large scale. The viral titre and multiplicity of infection (MOI) was determined in a 96-well plate according to the manufacturer's instructions. To further confirm the success of IDO gene transfection, the 293 transfected and untransfected cell lysates were prepared, DNA was extracted and the presence of IDO gene was confirmed by PCR analysis using sense (5′-GACTACAAGAAAGAGTACCA-3′) and antisense (5′-TTGGGTTA CATTAACCTTCC-3′) IDO gene primers.

The expression and biological activity of the IDO was evaluated by microscopic evaluation of GFP-positive cells and measuring the level of tryptophan and its degraded product, kynurenine, present in conditioned medium derived from IDO and control vector-infected cells. The conditioned medium related to the same number of cells was deproteinized by adding 100 μL of acetone to 50 μL of cell culture medium. It was vortexed for 30 s, cooled on ice for 5 min, and centrifuged at 16,000 g at 4°C for 15 min. The supernatant of each sample received 10 μL of 0.25 M $HClO_4$ and IS (4-amino-hippuric acid) solution and then the mixture was incubated at 25°C for 20 min and then dried under a vacuum. One hundred microliters of 14% BF_3-propanol was then added and the mixture was heated at 90°C for 2 h. The vacuum dried material was dissolved in 40 μL of 20% methanol in water and analyzed by liquid chromatography with electrospray mass spectrometry (LC/MS Agilent Technologies Canada, Inc., Mississauga, ON Canada) using a Hewlett Packard series 1100 mass selective detector controlled by 1100-MSD Chem. Station. The mass spectrometer was operated in positive ion mode. Tryptophan and kynurenine quantitative analyses were performed in the selected ion-monitoring mode.

Detection of IDO protein by western blotting For detection of IDO expression, non- (C), Ad-GFP- (V), or Ad-GFP-IDO- (IDO) infected cells were harvested after 72 h post-infection and washed twice with PBS. Cells were then lyzed in lysis buffer (50 mM Tris-HCl, pH 7.4, 10 mM EDTA, 5 mM EGTA, 0.5% NP40, 1% Triton X-100, and protease inhibitor cocktail (Sigma, Sigma Chemicals, Oakville, ON, Canada)). Cell lysate was centrifuged at 16,000 g for 15 min. The protein concentration of supernatant was measured by BCA protein assay kit (Pierce, Rockfield, IL). Equal amounts of total protein were separated by 12% SDS-PAGE and immunoblotted with our recently raised rabbit polyclonal IDO antibody at final concentration of 1:5000. Horseradish peroxidase-conjugated goat anti-rabbit IgG served as a secondary antibody for the enhanced chemiluminescence detection system (ECL; Amersham).

Viral infection and cell co-culture system Human fibroblasts, keratinocytes, and endothelial cells were separately infected with either Ad-IDO or control viral vectors for 30 h at an MOI of 2000, 100, and 100, respectively. The expression of IDO was confirmed by: (1) monitoring GFP expression under fluorescence microscopy (Nikon, Melville, NY HB-1010 AF); (2) measuring the level of kynurenine in conditioned medium; and (3) the detection of IDO protein expression using western blot. Infected cells were then washed twice with PBS to remove the viral particles and then sub-cultured in six-well plates for co-culture assays. Fibroblasts and immune cells were co-cultured into a two-chamber culture plate in which 0.4 μm insert (Millipore, Bedford, MA) separates the upper (fibroblasts) chamber from the bottom (immune cells). An RPMI 1640 culture medium containing 10% FBS and 2 mM glutamine, a suitable culture medium for both immune cells and fibroblasts, was used in this co-cultured assay. Immune cells were harvested at the different time points. Similarly, using a two-chamber co-culture system, either Ad-GFP- or Ad-IDO-GFP-infected cells were co-cultured with non-infected either keratinocytes or fibroblasts or endothelial cells for different durations. Cells (non-infected cells) from bottom chambers were harvested, stained and subsequently evaluated by fluorescence-activated cell sorting (FACS) analysis.

IFN treatment In another approach to induce IDO expression in dermal fibroblasts (Sarkhosh *et al*, 2003), cells were treated with 1000 U per mL of IFN-γ (GIBCO) or vehicle alone for 40 h to stimulate the expression of IDO to its maximal level. The cells were then washed twice with PBS to remove the IFN-γ and co-cultured with Jurkat cells for another 4 d. The IDO activity in fibroblasts induced by IFN was monitored by measuring the level of kyrurenine in conditioned medium as previously described.

Propidium iodide (PI) staining and flow-cytometric analysis Harvested cells were stained with 10 μg per mL of PI (Sigma) for 10 min. Stained cells were centrifuged and washed twice with fresh medium. Fluorescence microscopy (Nikon, HB-10101AF) was used to monitor the pattern of PI-stained cells and flow cytometry (FACScan, Becton-Dickinson, CO, Mountain View, CA) was used to quantify the number of PI-positive cells.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin Nick end labelling (TUNEL) Cyto-spinned cells were fixed with acetone:methanol (1:1) for 10 min at room temperature, washed three times with PBS, then subjected to TUNEL assay. The fixed cells were incubated in TdT buffer containing 30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, and 1 mM cobalt chloride for 10 min at room temperature. A mixture of bio-16-dUTP (Sigma) at 16.5 μM dATP (Boehringer Mannheim, Laval, QC, Canada), and TdT enzyme at 5 U per mL (Boehringer Mannheim) in TdT buffer was added and the reaction was allowed to proceed for 60 min at 37°C. The reaction was terminated by addition of 2 $\times$ SSC (300 mM sodium chloride plus 30 mM sodium citrate). After washing the cells three times in ddH_2O, endogenous peroxidase activity was quenched in a 3% H_2O_2 solution for 10 min at room temperature. Cells were rinsed with PBS and non-specific binding was reduced by incubation of cells with blocking buffer (3% skim milk powder in PBS containing 0.5% Tween 20) for 15 min. The cells were incubated for 30 min at room temperature with ExtrAvidin peroxidase (Sigma) diluted 1:50 in blocking buffer. Cells were washed three times with ddH_2O, and then stained with aminoethyl carbizole at room temperature until a red color appeared.

Cell proliferation of IDO-expressing skin cells [^{3}H]thymidine incorporation was employed to determine and compare the proliferation rate between IDO- and non-IDO-expressing cells. Fibroblasts and keratinocytes were infected with either nothing (control), Ad-GFP (vector), or Ad-GFP-IDO (IDO) for 30 h. Free viral particles were removed by washing with PBS and cells were allowed to grow until day 3 or 6. Cells then received 1 μCi per mL of [^{3}H]thymidine (Amersham/Pharmacia Biotech) for 16 h, harvested and washed three times with PBS. The radioactivity was determined by scintillation counting.

Statistics All values are presented as mean $\pm$ SD. The comparisons of cell proliferation between IDO- and non-IDO-expressing cells were assessed using the non-parametric Fisher's exact test. A p-value of <0.05 was considered statistically significant.

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